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Constitutive androstane receptor transcriptionally activates human *CYP1A1* and *CYP1A2* genes through a common regulatory element in the 5′-flanking region

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ABSTRACT

Phenobarbital has long been known to increase cellular levels of CYP1A1 and CYP1A2 possibly through a pathway(s) independent of aryl hydrocarbon receptor. We have investigated the role of constitutive androstane receptor (CAR), a xenobiotic-responsive nuclear receptor, in the transactivation of human *CYP1A1* and *CYP1A2*. These genes are located in a head-to-head orientation, sharing a 5′-flanking region. Reporter assays were thus performed with dual-reporter constructs, containing the whole or partially deleted human *CYP1A* promoter between two different reporter genes. In this system, human CAR (hCAR) enhanced the transcription of both genes through common promoter regions from −461 to −554 and from −18089 to −21975 of *CYP1A1*. With reporter assays using additional deleted and mutated constructs, electrophoresis mobility shift assays and chromatin immunoprecipitation assays, an ER8 motif (everted repeat separated by eight nucleotides), located at around −520 of *CYP1A1*, was identified as an hCAR-responsive element and a binding motif of hCAR/human retinoid X receptor α heterodimer. hCAR enhanced the transcription of both genes also in the presence of an aryl hydrocarbon receptor ligand. Finally, hCAR activation increased *CYP1A1* and *CYP1A2* mRNA levels in cultured human hepatocytes. Our results indicate that CAR transactivates human *CYP1A1* and *CYP1A2* in human hepatocytes through the common *cis*-element ER8. Interestingly, the ER8 motif is highly conserved in the *CYP1A1* proximal promoter sequences of various species, suggesting a fundamental role of CAR in the xenobiotic-induced expression of *CYP1A1* and *CYP1A2* independent of aryl hydrocarbon receptor.

1. Introduction

CYP1A1 and CYP1A2 are involved in the metabolic activation of procarcinogenic and hepatotoxic compounds as well as in the detoxification of drugs, nutrients, and environmental pollutants. These enzymes are highly inducible after exposure to polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This induction results from the enhancement of transcription of both genes through the activation of aryl hydrocarbon receptor (AhR) [1]. In response to ligand exposure, AhR translocates to the nucleus and heterodimerizes with AhR nuclear translocator. The heterodimer binds to the *cis*-element termed xenobiotic-responsive element (XRE) in the 5′-flanking region of target genes to activate their transcription. Human *CYP1A1* and *CYP1A2* are located in a head-to-head orientation in chromosome 15 and share a ~23 kb 5′-flanking region, in which twelve XREs are found. We have recently demonstrated that both genes share a common regulatory region and that the region from −461 to −1826 of *CYP1A1* containing five XREs works bidirectionally to enhance the transcription of human *CYP1A1* and *CYP1A2* [2,3]. Mouse and rat *CYP1A1* and *CYP1A2* are also located in a head-to-head orientation and share a corresponding 5′-flanking region. These data suggest the commonality of the AhR-XRE system for CYP1A1 and CYP1A2 induction in various species.

Constitutive androstane receptor (CAR), a member of nuclear receptor superfamily, plays a pivotal role in the drug-induced expression of CYP2A, CYP2B, CYP2C, and CYP3A subfamily enzymes, in concert with pregnane X receptor [4]. CAR is normally retained in cytoplasm and translocates to the nucleus in response to the exposure of drugs such as phenobarbital (PB). In nucleus, CAR heterodimerizes with retinoid X receptor α (RXRα), binds to *cis*-elements in the promoter region of target genes, and enhances their transcription.

Involvement of CAR in the drug-induced expression of *CYP1A1* and *CYP1A2* is also suggested. Treatment with PB resulted in the

Abbreviations: AhR, aryl hydrocarbon receptor; XRE, xenobiotic-responsive element; CYP, cytochrome P450; CAR, constitutive androstane receptor; RXRα, retinoid X receptor α; h, human; PB, phenobarbital; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; PCR, polymerase chain reaction; SEAP, secreted alkaline phosphatase; Luc, luciferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; ANOVA, analysis of variance.

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increased hepatic *Cyp1a2* mRNA levels in Ahr-null mice [5] and DBA/2 mice that are aryl hydrocarbon-nonresponsive [6]. The molecular mechanism, however, remains unclear. In the present study, we have investigated the role of CAR in the expression of *CYP1A1* and *CYP1A2* using the dual-reporter assay system recently developed in our laboratory [3]. We here demonstrate that human CAR (hCAR) transactivates human *CYP1A1* and *CYP1A2*, which is independent of AhR.

2. Materials and methods

2.1. Reagents

T4 polynucleotide kinase, alkaline phosphatase (*E. coli* C75) and restriction enzymes, unless otherwise stated, were purchased from New England BioLabs (Ipswich, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Nissui Pharmaceutical Co. (Tokyo, Japan) and BioWest (Nuaille, France), respectively. Media supplements were obtained from Invitrogen (Carlsbad, CA). β-Naphthoflavone, bovine serum albumin, poly(dI-dC), protease inhibitor cocktail, and proteinase K were purchased from Sigma–Aldrich (St. Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and [γ-^{32}P]ATP were obtained from Merck (Darmstadt, Germany) and PerkinElmer (Waltham, MA), respectively. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Oligonucleotides were commercially synthesized by Fasmac (Atsugi, Japan).

2.2. Plasmid DNA

Dual-reporter constructs used in Figs. 1 and 2 were prepared previously [3].

pd1AΔ−670/−21975 and pd1AΔ−883/−21975: pd-1A1/1A2 was digested with ApaI, and the resulting 10-kb fragment was ligated itself using DNA Ligation Kit (Takara Bio, Otsu, Japan). This was digested with HindIII to obtain ∼7-kb and ∼3-kb fragments (named F1 and F2, respectively). F2 was inserted into the HindIII site of pGEM-3Zf(+) (Promega, Madison, WI), and it was digested with Bst1107I (Takara Bio) and EcoO65I (Takara Bio) to prepare a DNA fragment (F3). Polymerase chain reaction (PCR) was carried out using *TaKaRa Ex* Taq (Takara Bio) to amplify regions from

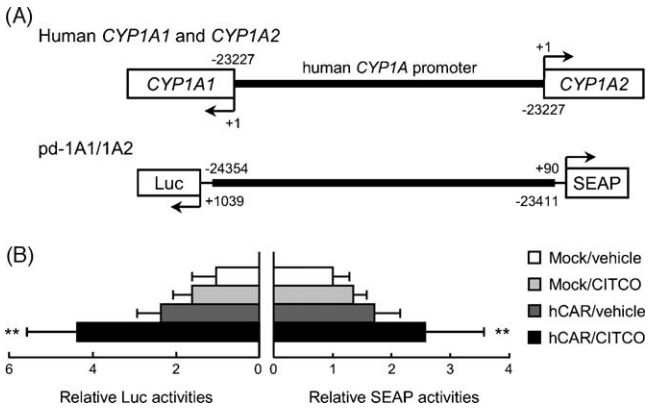

Fig. 1. Influence of hCAR activation on the transcription of *CYP1A* dual-reporter gene. (A) Schematic structures of human *CYP1A1* and *CYP1A2* and dual-reporter plasmid pd-1A1/1A2 are shown. The numbers above and below the gene/plasmid represent the positions from the transcriptional starting points of *CYP1A2* and *CYP1A1*, respectively. (B) Reporter assays were performed with HepG2 cells in 48-well plates as described in Section 2. Reporter activities normalized with β-galactosidase activities are shown as ratios to those in control cells (Mock/vehicle). Data are shown as mean ± SD ($n = 4$). $^{**}p < 0.01$ vs. Mock/vehicle (ANOVA followed by Dunnett's post-hoc test).

−455[1] to −669 or from −455 to −882 with human genome as a template and primers shown in Table 1. The amplicons were independently inserted into pCR2.1 using TOPO TA Cloning Kit (Invitrogen). These were digested with Bst1107I and EcoO65I and each resulting fragment was ligated to F3. The products were digested with PvuI and HindIII to obtain ∼3-kb fragments. These were independently ligated to F1 to obtain pd1AΔ−670/−21975 and pd1AΔ−883/−21975.

Constructs used in Figs. 3A, 3C, 4B and 7: Double-stranded oligonucleotides (Table 1) were ligated to F3, and the resulting plasmids were digested with Bst1107I and EcoO65I. The fragments yielded were ligated to F1 to obtain reporter constructs.

pd1AΔ−1827/−21975m: pd-1A1/1A2 was digested with NheI, and the resulting ∼4-kb fragment was inserted into pGL4.10-basic (Promega). With this plasmid as a template, a mutation was introduced using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and primers, 5′-TGCGACCCCAGCCCT-GATGTAACGGGGGCC-3′ and 5′-GGCCCCCGTTACATCAGGGCTGGG-GTCGCA-3′. The mutated plasmid was digested with NheI, and the resulting ∼4-kb fragment was inserted at the NheI site of pd1AΔ−1827/−21975.

Others: pcDNA3.1/V5-His-hCAR (pcDNA-hCAR) [7] was generous gift from Dr. Negishi (NIEHS/NIH, Research Triangle Park, NC). The hCAR cDNA was amplified by PCR using primers 5′-GCGGATCCCGTCATGGCCAGTAGGGAAGATG-3′ and 5′-GCAAGCTTTCAGCTGCAGATCTCCTGGAGCAG-3′ and inserted into pTargeT (Promega) to obtain pT-hCAR. The cDNA fragment obtained by digesting pT-hCAR with MluI and NotI was inserted into the same restriction sites of pTNT (Promega; pTNT-hCAR) for in vitro synthesis. pTNT-hRXRα was reported previously [8].

2.3. Reporter assays

HepG2 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FBS, minimum essential medium nonessential amino acids, and antibiotic–antimycotic. Cells were seeded in 24- or 48-well plate (BD Biosciences, Heidelberg, Germany) at 6×10^4 or 3×10^4 cells/well, respectively, 24 h before transfection. Each reporter plasmid and either pcDNA3.1 (Invitrogen) or pcDNA-hCAR were cotransfected using jetPEI (PolyPlus Transfection, Illkirch, France). pSV-β-galactosidase (Promega) was cotransfected to normalize transfection efficiency. Eight hours after transfection, cells were treated with 0.3 μM CITCO or vehicle (0.1% DMSO) for 40 h, or with 0.3 μM CITCO, 10 μM β-naphthoflavone and/or vehicle (0.2% DMSO) for 40 h (Fig. 8). Subsequently, aliquots of the medium were collected to measure secreted alkaline phosphatase (SEAP) activities with Great EscAPe SEAP Chemiluminescence Kit 2.0 (Clontech), and the cells were harvested to measure luciferase (Luc) and β-galactosidase activities as described previously [8].

2.4. Electrophoretic mobility shift assay (EMSA)

hCAR and hRXRα were synthesized in vitro with pTNT-hCAR and pTNT-hRXRα, respectively, using TNT Quick Coupled Transcription/Translation System (Promega). Control lysate was prepared using empty pTNT. Double-stranded oligonucleotides (Fig. 5A) were ^{32}P-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified with Nick columns (GE Healthcare, Buckinghamshine, UK). hCAR- or hRXRα-containing, or control lysates (1 μl each) were incubated with each ^{32}P-labeled probe (35 fmol) in reaction mixture (15 μl) containing 10 mM Tris–HCl, pH 8.0, 5%

[1] Nucleotide positions are numbered from the transcription initiation site of *CYP1A1* (NM_000499), unless otherwise stated.

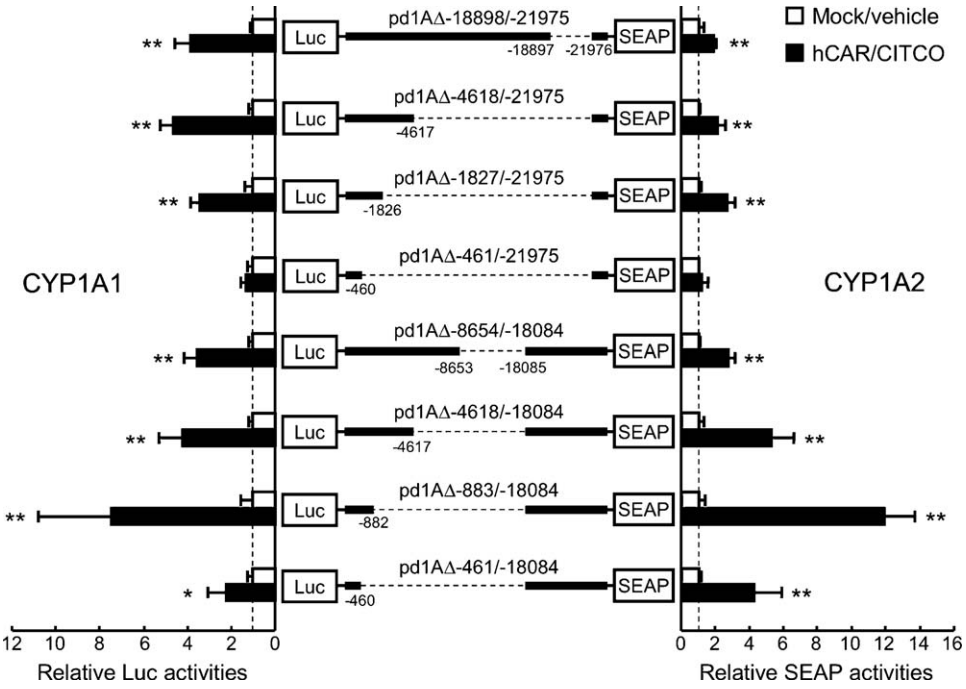

Fig. 2. Identification of hCAR-responsive region in human *CYP1A* promoter. Schematic structures of reporter plasmids used are shown in the middle. Reporter assays were carried out and the results are shown as in Fig. 1B. Data are the mean ± SD ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ vs. Mock/vehicle for each reporter plasmid (Student's t-test).

glycerol, 100 mM KCl, 1 mM dithiothreitol and 1 μg of poly(dI-dC) for 30 min at room temperature. For competition assays, unlabeled probes were added to the reaction mixture at various concentrations. Protein–DNA complexes were separated on 4% non-denaturing polyacrylamide gel in 0.25 × TBE buffer (22.5 mM Tris–HCl, 22.5 mM boric acid, 0.5 mM EDTA, pH 8.0) and detected with FLA-3000 Image analyzer (FujiFilm, Tokyo, Japan).

2.5. Chromatin immunoprecipitation assay

HepG2 cells were transfected with pcDNA-hCAR using jetPEI. Eight hours after transfection, the cells were treated with 0.3 μM CITCO for 24 h. Chromatin immunoprecipitation (ChIP) assays were carried out according to the protocol reported previously [9] with minor modifications. After crosslinking with 1% formaldehyde and sonication of cells, supernatant was obtained by centrifugation. Meanwhile, protein G-coupled Dynabeads (Invitrogen) were incubated with normal rabbit IgG (Millipore, Billerica, MA) or rabbit IgG against V5 (MBL, Nagoya, Japan) or RXRα (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. The chromatin-containing supernatant was immunoprecipitated with the Dynabeads-antibody complex at 4 °C overnight, and a portion of the supernatant was kept as an input sample. The bound chromatin was eluted and incubated at 65 °C overnight to decrosslink. After treatment with RNase A (Nacalai Tesque, Kyoto, Japan) and proteinase K, genomic DNA was purified with Wizard SV Gel and PCR Clean-Up System (Promega) and used as a template for PCR with *TaKaRa Ex* Taq Hot Start Version (Takara Bio). Primers

Table 1
Oligonucleotides used for plasmid construction.

Construct	Sense primer	Antisense primer
pd1AΔ−883/−21975	5′-GGTGACCTCCTTCCCGGGGTTA-3′	5′-GTATACGCTAGCCGGGGGTAGGGTGGGGGCTC-3′
pd1AΔ−670/−21975	5′-GGTGACCTCCTTCCCGGGGTTA-3′	5′-GTATACGAGGAGCCGCTAGTGAGCGCTCAG-3′
pd1AΔ−555/−21975	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGACCCCAGCCCTGAGGTCACGGGGGCCGGAAGGC-CAGAGGCGCCGCGGGTA-3′	5′-TACCCGCGGCGCCTCTGGCCTTCCGGCCCCCGTGA-CCTCAGGGCTGGGGTCGCAGCGCTTCTCACGCGAGCC-GGGACTCAGTAACCCCGGGAAGGAG-3′
pd1AΔ−511/−21975	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGACCGTA-3′	5′-TACGGTCGCAGCGCTTCTCACGCGAGCCGGGACTCA-GTAACCCCGGGAAGGAG-3′
pd1AΔ−498/−21975	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGT-GAGGTA-3′	5′-TACCTCACGCGAGCCGGGACTCAGTAACCCCGGGAA-GGAG-3′
pd1AΔ−555/−21975m1	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGACCCCAGCCCTGATGTAACGGGGGCCGGAAGGC-CAGAGGCGCCGCGGGTA-3′	5′-TACCCGCGGCGCCTCTGGCCTTCCGGCCCCCGTTACA-TCAGGGCTGGGGTCGCAGCGCTTCTCACGCGAGCCGGG-ACTCAGTAACCCCGGGAAGGAG-3′
pd1AΔ−555/−21975m2	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGACCCCATCCATGAGGTCACGGGGGCCGGAAGGC-CAGAGGCGCCGCGGGTA-3′	5′-TACCCGCGGCGCCTCTGGCCTTCCGGCCCCCGTGACC-TCATGGATGGGGTCGCAGCGCTTCTCACGCGAGCCGGG-ACTCAGTAACCCCGGGAAGGAG-3′
pd1AΔ−555/−21975m3	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGACCCCAGCCCTGAGGTCACGGGGGCCGGAATGC-AAGAGGCGCCGCGGGTA-3′	5′-TACCCGCGGCGCCTCTTGCATTCCGGCCCCCGTGACC-TCAGGGCTGGGGTCGCAGCGCTTCTCACGCGAGCCGGG-ACTCAGTAACCCCGGGAAGGAG-3′
pd1AΔ−555/−21975ΔER8	5′-GTGACCTCCTTCCCGGGGTTACTGAGTCCCGGCTCGCGTGAG-AAGCGCTGCGGGGGCCGGAAGGCCAGAGGCGCCGCGGGTA-3′	5′-TACCCGCGGCGCCTCTGGCCTTCCGGCCCCCGCAGCG-CTTCTCACGCGAGCCGGGACTCAGTAACCCCGGGA-AGGAG-3′

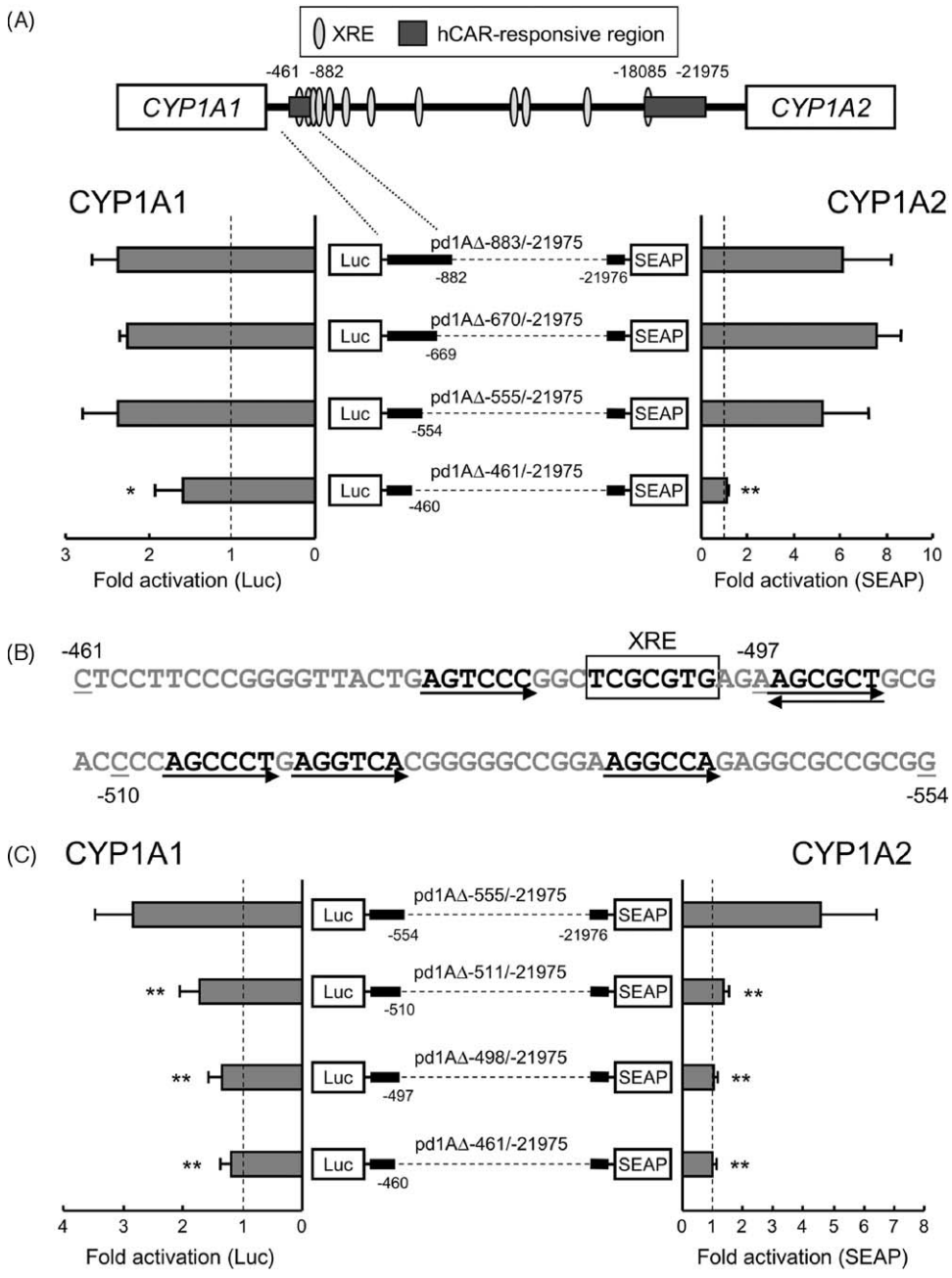

Fig. 3. Identification of hCAR-responsive region within the XRE cluster. (A and C) Schematic structures of human *CYP1A1* and *CYP1A2* and reporter plasmids used are shown on the top and middle, respectively. Reporter assays with HepG2 cells in 24-well plates were carried out as described in Section 2. Values are shown as ratios of reporter activities in pcDNA-hCAR-transfected and CITCO-treated cells to those in pcDNA3.1-transfected and vehicle-treated cells. Data are the mean ± SD of three (A) or four (C) independent assays ($n = 3$ in each assay). $^{*}p < 0.05$; $^{**}p < 0.01$ vs. pd1AΔ−883/−21975-transfected cells in (A) or pd1AΔ−555/−21975-transfected cells in (C) (ANOVA followed by Dunnett's post-hoc test). (B) Nucleotide sequence of the region from −461 to −554 of human *CYP1A1* is shown. Arrows and box represent putative nuclear receptor binding motifs and XRE, respectively.

used to detect the ER8-containing region (from −379 to −693) were 5′-GAACGCTGGGCGTGCAGATGCCTC-3′ and 5′-CACTAAGGCGATCCTAGAGGCTG-3′, and those for the negative control region (from +1248 to +1542) were 5′-CTTACAGAGAAAGTATTGCCTCAG-3′ and 5′-GATAGGGGACAGGATGGCCACTTC-3′.

2.6. Measurement of mRNA levels in human hepatocytes

Cryopreserved human hepatocytes (lot. H817: white, female, 31-year-old), purchased from XenoTech (Lenexa, KS), were thawed and cultured as described previously [8]. The hepatocytes were infected

Table 2
Primers used for quantitative RT-PCR.

Gene	Forward	Reverse
CYP1A1	5′-AGGAGCTAGACACAGTGATTG-3′	5′-GTTCAGGTAGGAACTCAGATG-3′
CYP1A2	5′-GGCTTCTACATCCCCAAGAAATG-3′	5′-GCTGAACTCCAGTTGCTGTAG-3′
CYP2B6	5′-CATCATCCCCAAGGACACAG-3′	5′-AAATCCGCTTCCCTAAGGAG-3′
β-Actin	5′-GCCAACACAGTGCTGTCTG-3′	5′-CCTGCTTGCTGATCCACATC-3′

with hCAR-expressing adenovirus AdhCAR [10] at 0, 3 or 10 MOI. Total MOI was adjusted to 10 with empty adenovirus [10]. Forty-eight hours after infection, the cells were treated with 0.5 μM CITCO or vehicle (0.1% DMSO) for 24 h, and total RNA was isolated using Sepasol RNA I (Nacalai Tesque). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and primers shown in Table 2 with ABI7000 (Applied Biosystems).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett's post-hoc test and Student's *t*-test were performed using Prism version 4.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Transcriptional activation of CYP1A reporter genes by hCAR

In this study, we have used the dual-reporter system, which was recently developed in our laboratory [3]. Thus, transcriptional activities of *CYP1A1* and *CYP1A2* are simultaneously determined as Luc and SEAP activities, respectively. In a system using the dual-reporter construct pd-1A1/1A2, which contained the whole 5′-flanking region (Fig. 1A), cotransfection of hCAR-expressing plasmid (pcDNA-hCAR) slightly increased both Luc and SEAP activities, and CITCO treatment further increased both reporter activities in HepG2 cells (Fig. 1B). These results suggested that hCAR transactivated the expression of both human *CYP1A1* and *CYP1A2* through the 5′-flanking region bidirectionally.

To identify a CAR responsive region, reporter assays were carried out with various deletion constructs of pd-1A1/1A2. As shown in Fig. 2, both reporter activities of all the constructs except pd1AΔ−461/−21975 were increased with hCAR overexpression and CITCO treatment. Comparison of the results with pd1AΔ−1827/−21975 and pd1AΔ−461/−21975 showed that the deletion of nucleotides from −461 to −1826 resulted in complete loss of the hCAR-mediated expression of both reporter genes. Comparison of the results with pd1AΔ−883/−18084 and pd1AΔ−461/−18084 revealed that the deletion of nucleotides from −461 to −882 resulted in clear reduction in the hCAR-mediated expression of both genes. Comparison of the results with pd1AΔ−461/−21975 and pd1AΔ−461/−18084 revealed that the hCAR-mediated expression of both genes was completely abolished with the deletion of nucleotides from −18085 to −21975.

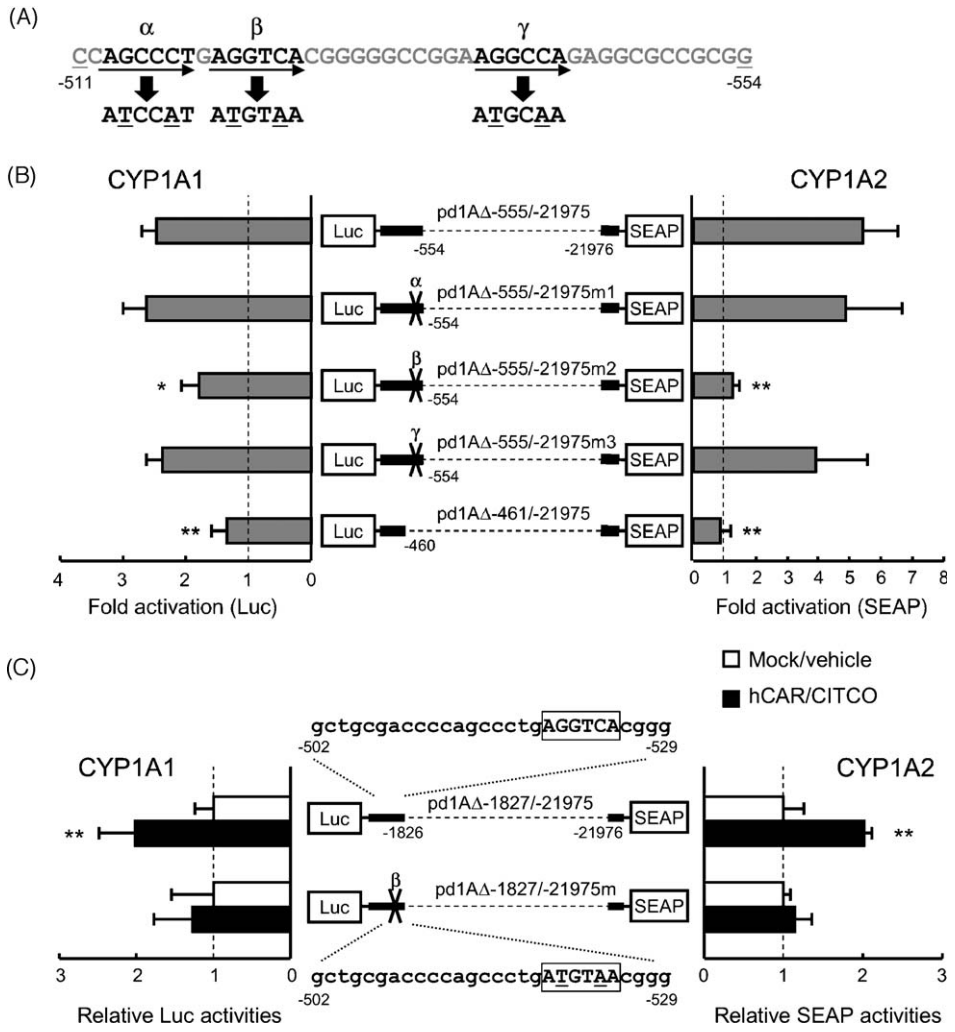

Fig. 4. Role of putative binding motifs for nuclear receptors in the hCAR-mediated transcription of *CYP1A* reporter genes. (A) Wild-type and mutated sequence of putative nuclear receptor binding half-sites (termed α, β, and γ) are shown. Mutated nucleotides are underlined. (B) Reporter assays with HepG2 cells in 24-well plates were carried out and the results are shown as in Fig. 3A. Data are the mean ± SD of four independent assays ($n = 3$ in each assay). $^{*}p < 0.05$; $^{**}p < 0.01$ vs. pd1AΔ−555/−21975-transfected cells (ANOVA followed by Dunnett's post-hoc test). (C) Schematic structures of reporter plasmids used are shown in the middle. Mutated nucleotides are underlined. Reporter assays with HepG2 cells in 48-well plates were carried out and the results are shown as in Fig. 1B. Data are the mean ± SD ($n = 4$). $^{**}p < 0.01$ vs. corresponding controls (Student's t-test).

These results suggested that two regions from −461 to −882 and from −18085 to −21975 were responsible for the hCAR-mediated transcriptional activation of both human *CYP1A1* and *CYP1A2*.

3.2. Identification of hCAR-responsive motif

Recently, we have shown that the XRE cluster, a region from −461 to −1826 of *CYP1A1*, is commonly essential for the AhR-mediated transcription of both human *CYP1A1* and *CYP1A2* [2,3]. Because XRE clusters are also found in other species such as rat and mouse and one of the hCAR-responsive regions (from −416 to −882) is located within the XRE cluster, we have focused on this region in this study. Several reporter constructs partially lacking this region were prepared for reporter assays. As shown in Fig. 3A, the deletion of nucleotides from −555 to −882 had no effect on both reporter activities while further deletion of nucleotides to −461 completely abolished the hCAR-mediated expression of SEAP gene and significantly reduced that of Luc gene. In this region, there are several putative binding half-sites for nuclear receptors, AG(G/T)TCA-related sequences (Fig. 3B). To examine the role of these motifs, deletion constructs were prepared for reporter assays. As shown in Fig. 3C, the deletion of nucleotides from −511 to −554 completely abolished the hCAR-mediated expression of SEAP gene and significantly reduced that of Luc gene. These results suggest that a hCAR-responsive element is located between −511 and −554.

To identify an essential half-site for the hCAR-mediated expression of *CYP1A1* and *CYP1A2*, three putative half-sites (termed α, β and γ) found in this region were independently mutated (Fig. 4A), and reporter assays were performed (Fig. 4B). The introduction of a mutation into α or γ half-site had no influence on the hCAR-mediated expression of both reporter genes. In contrast, a mutation of β half-site resulted in significant reduction in the hCAR-mediated Luc gene expression and complete loss of that of SEAP gene.

To confirm the role of the β half-site in the hCAR-mediated activation, another mutated construct pd1AΔ−1827/−21975m was prepared for reporter assays. As expected, the hCAR-mediated expression of both reporter genes was completely lost with this construct (Fig. 4C).

3.3. Identification of hCAR/hRXRα heterodimer binding motif

CAR forms a heterodimer with RXRα to bind to a DNA element that consists of two nuclear receptor binding half-sites. To identify a pair of half-site necessary for the binding of hCAR/hRXRα heterodimer, we carried out EMSA using various length probes containing the β half-site in combination with in vitro synthesized hCAR and hRXRα (Fig. 5A). As shown in Fig. 5B, hCAR/hRXRα bound to probes 8 and 9 with high affinity. Both probes contained an ER8-type motif, an everted repeat of two half-sites separated by eight nucleotides, which was suggested as a CAR/RXR binding element [11]. To confirm the binding of hCAR/hRXRα to ER8, EMSA was performed using probe 8m1, 8m2 and 8dm, which contained a mutation(s) in each half-site or both half-sites of ER8. As results, hCAR/hRXRα did bind to neither probe 8m1 nor 8dm, although the heterodimer faintly bound to probe 8m2 (Fig. 5B).

The binding specificity of hCAR/hRXRα to ER8 was further examined. As shown in Fig. 5C, only the heterodimer but not hCAR or hRXRα alone bound to probe 8. The shifted complex was

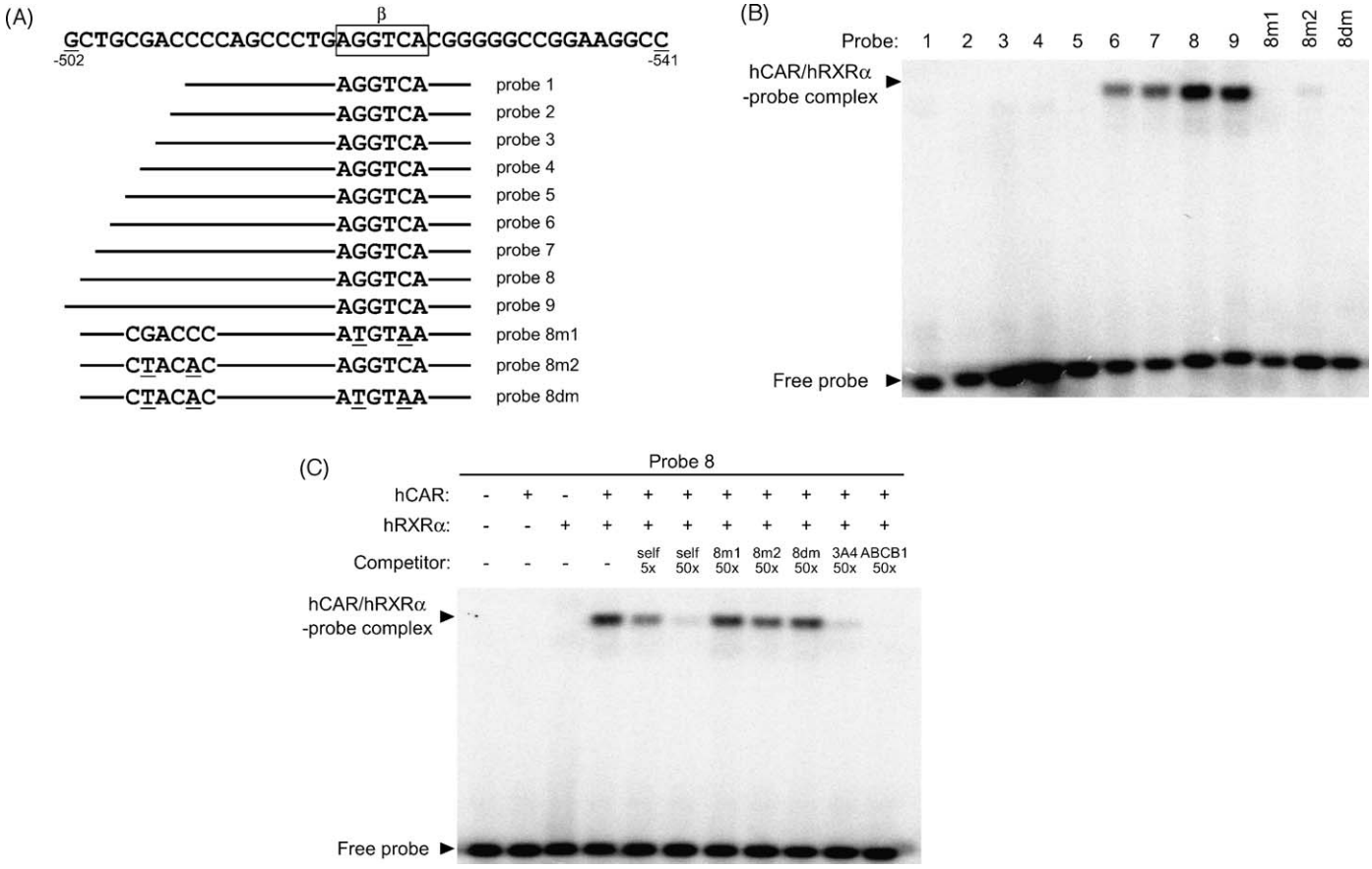

Fig. 5. Identification of hCAR/hRXRα binding motif. EMSA was carried out as described in Section 2 with radiolabeled oligonucleotides (A) and in vitro synthesized hCAR and/or hRXRα. In (A), bars represent the same nucleotides in *CYP1A1* promoter shown above. Mutated nucleotides are underlined. In (C), unlabeled oligonucleotides were added to reactions as competitors at the ratios indicated: self, probe 8; 3A4, *CYP3A4* prER6; ABCB1, human *ABCB1* DR4(I).

competed out by unlabeled probe 8 in a dose-dependent manner and by a 50-fold excess of both prER6 in human *CYP3A4* promoter and DR4(I) in human *ABCB1* promoter, both of which were reported as CAR binding sites [12,13], but not by either probe 8m1, 8m2 or 8dm (Fig. 5C).

To confirm the binding of hCAR/hRXRα to ER8 in a genomic context, ChIP assays were carried out with HepG2 cells transfected with pcDNA-hCAR and treated with CITCO. In this study, an antibody against a V5-tag was used to immunoprecipitate hCAR–chromatin complex because the V5-tagged hCAR was expressed. As depicted in Fig. 6, specific binding of hCAR and hRXRα to the *CYP1A1* promoter region containing ER8 (from −379 to −693), but not to a negative control region (+1248 to +1542), was detected.

To confirm the role of the ER8 motif identified in the hCAR-mediated transcription of human CYP1A1 and CYP1A2, a reporter construct lacking ER8 was prepared. As shown in Fig. 7, the deletion of ER8 drastically reduced the hCAR-mediated expression of both Luc and SEAP genes to levels similar to those of pd1AΔ−461/−21975.

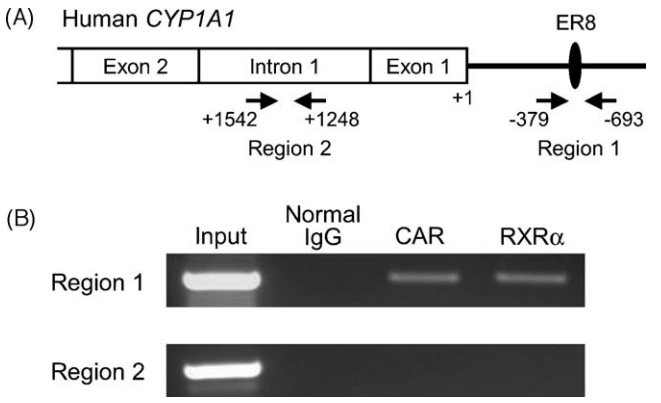

Fig. 6. Binding of hCAR/hRXRα to ER8 in human genome. (A) Schematic structure of human *CYP1A1* and the regions amplified in ChIP assays are shown. (B) ChIP assays were performed as described in Section 2. The precipitated DNA, along with the DNA isolated before immunoprecipitation (Input), were analyzed by PCR with specific primers for the region from −379 to −693 (Region 1) or region from +1248 to +1542 (Region 2; negative control) of human *CYP1A1*.

3.4. Independent transactivation of human CYP1A1 and CYP1A2 by hCAR and AhR

The fact that ER8 is located within the XRE cluster has raised the possibility of functional cooperation between AhR and CAR in the expression of human *CYP1A1* and *CYP1A2*. This possibility was investigated in reporter assays with two different constructs (Fig. 8). Both Luc and SEAP activities with the constructs were increased after individual activation of AhR through β-naphtho-

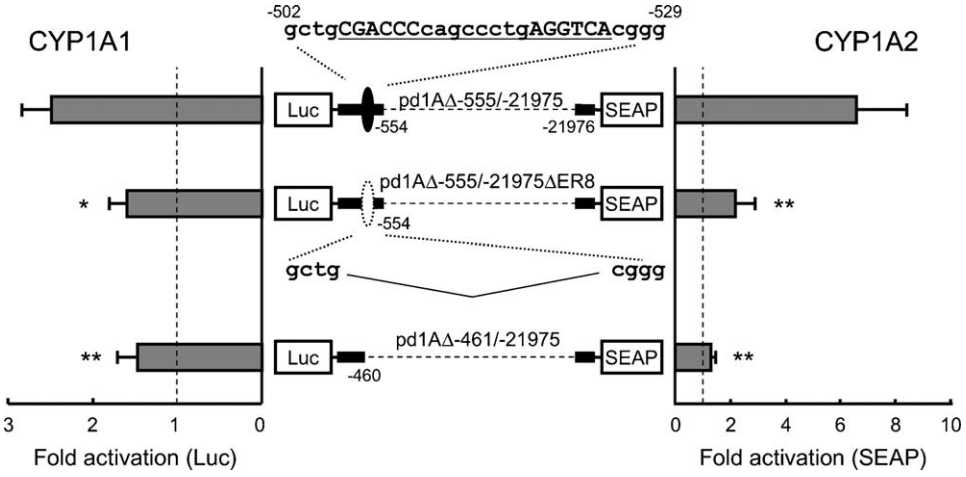

Fig. 7. Influence of the deletion of ER8 on the hCAR-mediated transcription of *CYP1A* reporter gene. Schematic structures of the reporter plasmids used are shown in the middle. Underlined nucleotides consist of ER8. Reporter assays with HepG2 cells in 24-well plates were carried out and the results are shown as in Fig. 3A. Data are the mean ± SD of three independent assays ($n = 3$ in each assay). $^{*}p < 0.05$; $^{**}p < 0.01$ vs. pd1AΔ−555/−21975-transfected cells (ANOVA followed by Dunnett's post-hoc test).

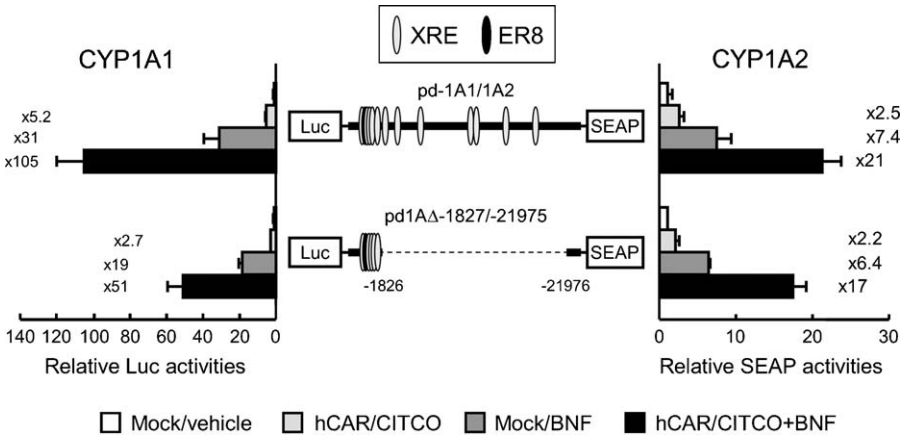

Fig. 8. Co-regulation of human *CYP1A* reporter genes by hCAR and AhR. Schematic structures of reporter constructs used are shown in the middle. Reporter assays with HepG2 cells in 48-well plates were performed as described in Section 2 and the results are shown as in Fig. 1B. Numbers on the left or right of the bars indicate fold-activations vs. control cells (Mock/vehicle). Data are the mean ± SD ($n = 4$). BNF, β-naphthoflavone.

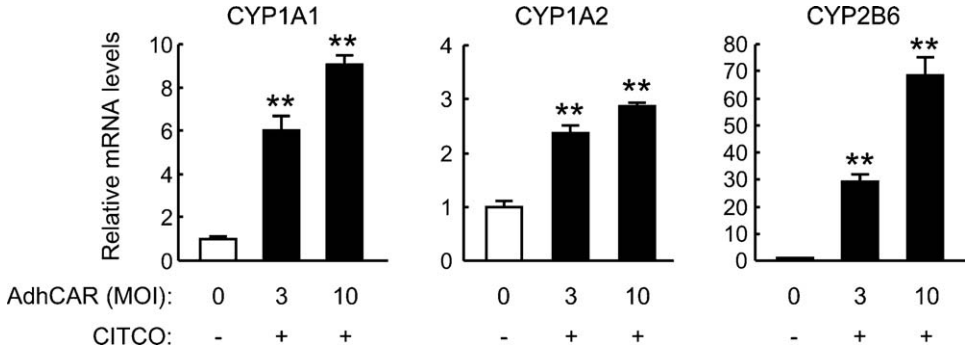

Fig. 9. Changes in *CYP1A1* and *CYP1A2* mRNA levels after hCAR activation in human hepatocytes. Human hepatocytes were infected with AdhCAR and/or control adenovirus and were treated with CITCO (0.5 μM) or vehicle (0.1% DMSO) for 24 h. Quantitative RT-PCR analyses were performed as described in Section 2. Relative mRNA levels normalized with β-actin mRNA levels are shown as the ratio to those in control cells (open bars). One representative result of two independent experiments is shown. Data are the mean ± SD ($n = 4$). $^{**}p < 0.01$ vs. control cells (ANOVA followed by Dunnett's post-hoc test).

flavone treatment or CAR through ectopic hCAR expression and CITCO treatment. In addition, hCAR activation further increased both reporter activities in the presence of an AhR ligand β-naphthoflavone.

3.5. Influence of hCAR activation on CYP1A1 and CYP1A2 mRNA levels in human hepatocytes

To investigate whether CAR activation increased *CYP1A1* and *CYP1A2* mRNA levels in human hepatocytes, cryopreserved human hepatocytes were infected with hCAR-expressing adenovirus AdhCAR and treated with CITCO, and then CYP mRNA levels were determined (Fig. 9). The mRNA level of *CYP2B6*, a well-known CAR target gene, was increased in the adenovirus concentration-dependent manner. Under this condition, both *CYP1A1* and *CYP1A2* mRNA levels were also increased 9.0-fold and 2.9-fold, respectively, after AdhCAR infection (10 MOI) with CITCO treatment.

4. Discussion

In the present study, we have investigated the molecular mechanism of the hCAR-mediated expression of human *CYP1A1* and *CYP1A2* in in vitro reporter assays with the dual-reporter system. The results with various deletion and mutated constructs demonstrate that hCAR enhances the transcription of both *CYP1A1* and *CYP1A2* through the ER8 motif located in the proximal promoter of *CYP1A1*. EMSA and ChIP assays have confirmed the binding of heterodimer of hCAR and hRXRα to ER8. The results obtained in reporter assays using an AhR ligand β-naphthoflavone suggest that the hCAR-mediated expression of *CYP1A1* and *CYP1A2* is independent of AhR, despite that ER8 is located within the XRE cluster which was demonstrated to be critical for the AhR-dependent transactivation of both genes [2,3]. Furthermore, hCAR activation in human hepatocytes increased mRNA levels of *CYP1A1* and *CYP1A2* as well as *CYP2B6*. CAR may also bind to the region from −18085 to −21975 and the role of this region remains to be elucidated in future studies. Nevertheless, our present results clearly indicate that hCAR transactivates both *CYP1A1* and *CYP1A2* in human livers in an AhR-independent manner through the ER8 motif located in the *CYP1A1* proximal promoter.

XRE clusters are well conserved in the proximal promoter regions of human, rat and mouse CYP1A1 (data not shown). Interestingly, we have found that the sequence of the ER8 motif, in addition to that of a nearby XRE, is also highly conserved in the proximal promoter region of *CYP1A1* among various species (Fig. 10). These suggest that the function of CAR–ER8 system is also conserved in these species for the expression of *CYP1A1* and *CYP1A2*. In fact, we have observed that hepatic *Cyp1a1* and *Cyp1a2* mRNA levels were increased after treatment of mice with a specific murine CAR activator 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene as well as PB (Yoshinari et al., unpublished results). Moreover, in our preliminary studies, no or little increase in Cyp1a1 and Cyp1a2 mRNA levels was observed in the liver of Car-null mice after 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene treatment (Yoshinari et al., unpublished results). Taken together, it is strongly suggested that the molecular mechanism for the gene expression of *CYP1A1*

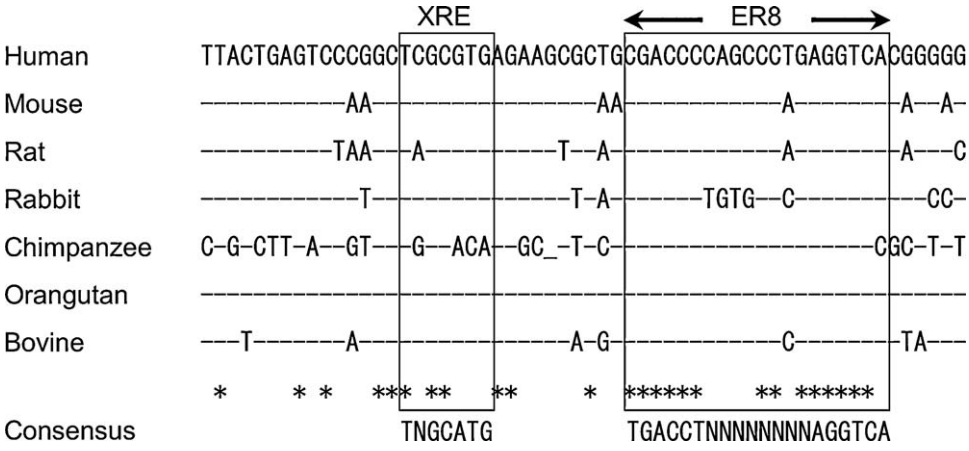

Fig. 10. Sequence comparison of the proximal promoters of *CYP1A1* of various species. The sequences were aligned by use of CLUSTALW on the DDBJ website. Only the nucleotides different from those in the human gene are shown and asterisks indicate the conserved nucleotides. N in the consensus sequences and an underscore in chimpanzee sequence represent any nucleotide and gap, respectively.

and *CYP1A2* in response to xenobiotics involving CAR as well as that involving AhR is conserved among various species.

In the present study, we have found the colocalization of hCAR/hRXRα binding element (ER8) very close to XRE in *CYP1A* genes. Adjacent localization of CAR and AhR binding sites is also found in human UDP-glucuronosyltransferase 1A1, *UGT1A1*. In the case of *UGT1A1*, hCAR/hRXRα binds to gtNR1, a direct repeat of two half-sites separated by four nucleotides, within the 290-bp PB-responsive enhancer module of *UGT1A1*, gtPBREM [14]. This region contains at least one XRE [15]. These facts thus suggest that the colocalization of CAR and AhR responsive elements is not restricted to *CYP1A1* and *CYP1A2* as a common regulatory system, although it remains to be investigated in future studies.

The present results imply a novel mechanism of drug–drug interactions through CYP1A1/CYP1A2 induction. In fact, there are several reports on interactions between hCAR-activating drugs such as phenytoin [16] and carbamazepine [17] and drugs that are metabolized by human CYP1A2. Phenytoin treatment decreased the plasma concentration of co-administered antipsychotic clozapine, a human CYP1A2 substrate [18]. A substantial decrease in the clozapine concentration was also observed in patients co-treated with carbamazepine compared to those treated with clozapine alone [19]. Another CYP1A2 substrate olanzapine was cleared more rapidly in subjects pretreated with carbamazepine than in control subjects [20]. Carbamazepine treatment also increased caffeine metabolism assessed by caffeine breath test in young patients [21]. Moreover, microarray analyses demonstrated increased mRNA levels of *CYP1A1* and *CYP1A2*, as well as *CYP2B6* and *CYP3A4*, in livers from epileptic patients treated with carbamazepine [22]. Although it is uncertain whether phenytoin and carbamazepine activate AhR, in addition to hCAR, or not, our present results are consistent with the possibility that the abovementioned drug–drug interactions result from CYP1A2 induction through the activation of CAR with the antiepileptic drugs.

In conclusion, we have identified the ER8 motif in the proximal promoter region of human *CYP1A1* as a binding site for hCAR/hRXRα heterodimer and demonstrated that the ER8 motif works bidirectionally for the hCAR-mediated transcription of both *CYP1A1* and *CYP1A2*. These findings indicate that *CYP1A1* and *CYP1A2* are new members of CAR target genes and may have important clinical implications for drug–drug interactions associated with CAR-mediated CYP induction. To our knowledge, this is the first report showing that a xenobiotic-activating nuclear receptor transactivates both *CYP1A1* and *CYP1A2*.

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References

[1] Whitlock Jr JP. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 1999;39:103–25.
[2] Yoshinari K, Ueda R, Kusano K, Yoshimura T, Nagata K, Yamazoe Y. Omeprazole transactivates human CYP1A1 and CYP1A2 expression through the common regulatory region containing multiple xenobiotic-responsive elements. Biochem Pharmacol 2008;76:139–45.
[3] Ueda R, Iketaki H, Nagata K, Kimura S, Gonzalez FJ, Kusano K, et al. A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. Mol Pharmacol 2006;69:1924–30.
[4] Sueyoshi T, Negishi M. Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. Annu Rev Pharmacol Toxicol 2001;41:123–43.
[5] Zaher H, Yang TJ, Gelboin HV, Fernandez-Salguero P, Gonzalez FJ. Effect of phenobarbital on hepatic CYP1A1 and CYP1A2 in the Ahr-null mouse. Biochem Pharmacol 1998;55:235–8.
[6] Sakuma T, Ohtake M, Katsurayama Y, Jarukamjorn K, Nemoto N. Induction of CYP1A2 by phenobarbital in the livers of aryl hydrocarbon-responsive and -nonresponsive mice. Drug Metab Dispos 1999;27:379–84.
[7] Kobayashi K, Sueyoshi T, Inoue K, Moore R, Negishi M. Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. Mol Pharmacol 2003;64:1069–75.
[8] Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K, et al. Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. Mol Pharmacol 2009;75:677–84.
[9] Lee TI, Johnstone SE, Young RA. Chromatin immunoprecipitation and microarray-based analysis of protein location. Nat Protoc 2006;1:729–48.
[10] Kamiyama Y, Matsubara T, Yoshinari K, Nagata K, Kamimura H, Yamazoe Y. Role of human hepatocyte nuclear factor 4alpha in the expression of drug-metabolizing enzymes and transporters in human hepatocytes assessed by use of small interfering RNA. Drug Metab Pharmacokinet 2007;22:287–98.
[11] Frank C, Gonzalez MM, Oinonen C, Dunlop TW, Carlberg C. Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. J Biol Chem 2003;278:43299–310.
[12] Burk O, Arnold KA, Geick A, Tegude H, Eichelbaum M. A role for constitutive androstane receptor in the regulation of human intestinal MDR1 expression. Biol Chem 2005;386:503–13.
[13] Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M. The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. J Biol Chem 1999;274:6043–6.
[14] Sugatani J, Kojima H, Ueda A, Kakizaki S, Yoshinari K, Gong QH, et al. The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. Hepatology 2001;33:1232–8.
[15] Yueh MF, Huang YH, Hiller A, Chen S, Nguyen N, Tukey RH. Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. J Biol Chem 2003;278:15001–6.
[16] Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M, LeCluyse E. Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. J Biol Chem 2004;279:29295–301.
[17] Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, et al. Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. J Pharmacol Exp Ther 2007;320:72–80.
[18] Miller DD. Effect of phenytoin on plasma clozapine concentrations in two patients. J Clin Psychiatry 1991;52:23–5.
[19] Jerling M, Lindstrom L, Bondesson U, Bertilsson L. Fluvoxamine inhibition and carbamazepine induction of the metabolism of clozapine: evidence from a therapeutic drug monitoring service. Ther Drug Monit 1994;16:368–74.
[20] Lucas RA, Gilfillan DJ, Bergstrom RF. A pharmacokinetic interaction between carbamazepine and olanzapine: observations on possible mechanism. Eur J Clin Pharmacol 1998;54:639–43.
[21] Parker AC, Pritchard P, Preston T, Choonara I. Induction of CYP1A2 activity by carbamazepine in children using the caffeine breath test. Br J Clin Pharmacol 1998;45:176–8.
[22] Oscarson M, Zanger UM, Rifki OF, Klein K, Eichelbaum M, Meyer UA. Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. Clin Pharmacol Ther 2006;80:440–56.